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Real-Time PCR Assay for Rapid Detection of Epidemiologically and Clinically Significant *Mycobacterium tuberculosis* Beijing Genotype Isolates

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***Mycobacterium tuberculosis* Beijing genotype strains are rapidly disseminating, frequently hypervirulent, and multidrug resistant. Here, we describe a method for their rapid detection by real-time PCR that targets the specific IS*6110* insertion in the *dnaA-dnaN* genome region. The method was evaluated with a geographically and genetically diverse collection representing areas in East Asia and the former Soviet Union in which the Beijing genotype is endemic and epidemic (i.e., major foci of its global propagation) and with clinical specimens.**

Mycobacterium tuberculosis is the major killer of humans in terms of morbidity and mortality. A closer look at its population structure reveals that different strains (lineages) of *M. tuberculosis* differ in their pathogenic capacities. The *M. tuberculosis* Beijing genotype is globally widespread and considered to be a fast-propagated family within *M. tuberculosis* (see references 1–3 and references therein). In Russia, the Beijing genotype strains are marked by increased virulence, as shown in a macrophage model (4), and are associated with multidrug resistance (5, 6). A "lethal combination" of the Beijing genotype and the human DC-SIGN −336G allele has been suggested for the male subgroup in the Siberian Slavic population (7). The capacity of the Beijing strains to escape a protection effect of the *Mycobacterium bovis* BCG vaccination in mice (8) suggests the clinical relevance of the early detection of this genotype.

For the above reasons, the availability of a simple method for detecting these strains is important for early adequate treatment and for epidemiological monitoring of their circulation. While no single standardized method for detecting the Beijing genotype has been approved, the availability of a wide array of methods would be helpful in view of the different technical capacities in different laboratories.

Previously, we reported the utility of the IS*6110*-based inverse PCR method for detecting Beijing strains based on specific and easily recognizable double-band profiles in agarose gel (9). One of these bands represents an IS*6110* insertion in the *dnaA-dnaN* region in the proximity of *oriC* (position 1592 in the H37Rv genome [our unpublished data]), also previously described by Kurepina et al. (10). Here, we developed a method to detect this Beijing genotype-specific insertion, *dnaA-dnaN*::IS*6110*, in a real-time PCR format and evaluated it on a geographically and genetically diverse collection of 724 isolates from the main areas across Eurasia (Russia, Belarus, Kazakhstan, China, Vietnam, Japan) in which the Beijing genotype is epidemic and endemic. In addition, the method was preliminarily tested on DNA extracted from clinical specimens.

Real-time PCR with three primers and two labeled probes was performed in a single tube in multiplex format targeting the same genomic region and detecting its two alleles, *dnaA-dnaN*::IS*6110* specific for the Beijing genotype and the intact *dnaA-dnaN* region. Under this design, a PCR product should always be amplified with either one or another pair of primers and should consequently give rise to one or another fluorescent signal indicating either the Beijing or another genotype. The interpretation of the assay result is based on comparison of the two fluorescence curves for the same isolate, i.e., presence of a signal in one channel and the absence of a signal in another channel.

DNA was extracted from a bacterial culture using a recommended method (11). Purified DNA (0.5 to 1 ng) was added to the PCR mix (final volume, 25 μl) containing 1.5 mM $MgCl_2$, 1 U *TaqF* hot-start DNA polymerase (InterLabService, Russia), 200 μM (each) deoxynucleoside triphosphates (dNTP), 4 pmol each of the primers BGR (5′-CGCCGGGACTGTATGAGTCT), BGF2 (5′-CTCTCCCAGGTCACACCAGTCA), and BGRi (5′-TCGATGAACCACCTGACATGAC), and the probes BGPi (FAM-5′-CGGCATGTCCGGAGACTCCAGTTC [where FAM is 6-carboxyfluorescein], 1.5 pmol) and BGP2 (HEX-5′-TGGCTGTGAGTGTCGCTGTGCACA [where HEX is hexachloro fluorescein], 2.5 pmol). The PCR was run in a Rotor-Gene 6000 (Corbett Research) under the following conditions: 95°C for 10 min, 30 cycles at 94°C for 15 s, and 60°C for 50 s. Signal detection was performed at 60°C. A Beijing-specific insertion, *dnaA-dnaN*::IS*6110*, was revealed using the primers BGF2 and BGRi and the probe BGPi (green detection channel, wavelength of 510 nm). The intact

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dnaA-dnaN region was revealed using the primers BGF2 and BGR and the probe BGP2 (yellow detection channel, wavelength of 555 nm).

The Latin-American Mediterranean (LAM) family of *M. tuberculosis* was detected by a MboII PCR-restriction fragment length polymorphism (RFLP) analysis of *Rv0129c* 309 G→A mutation (12). The East-African Indian (EAI) family of *M. tuberculosis* was detected based on PCR detection of the RD239 deletion (13). To negate the possibility of a "pseudo-Beijing" genotype (14), the Beijing genotype isolates used in the initial optimization step in this study were confirmed to belong to the Beijing genotype by RD207 deletion analysis (15).

DNA from clinical specimens obtained from tuberculosis patients was extracted using commercial kits employing magnetic sorbent-based technology (AmpliSens [InterLabService] or M-Sorb-TUB [Syntol, Moscow, Russia]). Real-time PCR analysis of these samples was done as described above with a modification: 20 μl of DNA was added to a total volume of 50 μl PCR mix, and the signal accumulation was evaluated within 50 cycles. PCR inhibition was checked using a control assay implemented in the commercial kit to detect *M. tuberculosis* complex (AmpliTub-RV; Syntol), while an internal control (artificial DNA fragment cloned in the pUC19 plasmid) was amplified and detected in another fluorescence channel. In clinical samples, the concentrations of DNA were estimated using control samples and a calibration assay implemented in the same kit.

The assay was optimized using DNA of non-Beijing strains (including the reference strains H37Rv and BCG) and of Beijing strains of *M. tuberculosis* previously characterized using spoligotyping and confirmed to belong to the Beijing genotype by presence of the RD207 deletion.

Accumulation of the Beijing-specific signal was registered in the channel FAM/green (Fig. 1a), and accumulation of the non-Beijing genotype signal was registered in the channel HEX/yellow (Fig. 1B). The presence of Beijing DNA in a given sample was manifested as an exponential increase of the Beijing channel FAM signal and with the complete lack of signal in the non-Beijing channel HEX.

The optimized method was further evaluated with a representative and diverse (based on IS*6110*-RFLP and/or 24-loci mycobacterial interspersed repetitive-unit–variable-number tandem-repeat [MIRU-VNTR] typing and spoligotyping) collection of 724 DNA samples of *M. tuberculosis* isolates from the main areas of Beijing genotype endemicity in the former Soviet Union (Russia, Belarus, Kazakhstan) and East Asia (China, Vietnam, Japan) previously characterized by spoligotyping, IS*6110*-RFLP, and/or 24-MIRU-VNTR typing (see Table S1 and S2 in the supplemental material). This analysis revealed a complete concordance of the two methods in detecting the Beijing strain (real-time PCR and spoligotyping), with a few exceptions detailed below. Four hundred sixty-two isolates with a complete nine-signal spoligoprofile or abridged Beijing-like profiles with deleted signals were correctly identified as the Beijing genotype. In turn, 252 non-Beijing strains (genetic families LAM [$n = 96$], Haarlem [$n = 26$], Ural [$n = 20$], T [$n = 66$], X [$n = 2$], EAI [$n = 25$], unknown family [$n = 17$]) were correctly identified as non-Beijing.

The discrepant cases, showing the simultaneous presence of Beijing and non-Beijing real-time PCR signals, concerned 9 isolates from the former Soviet Union countries and 1 isolate from Japan with non-Beijing spoligotypes. A closer look at their spoligoprofiles revealed the presence of the 9 last signals characteristic of the Beijing genotype. Accordingly, these DNA samples were tested for additional markers that define other *M. tuberculosis* families. As a result, it was found that 9 isolates from countries in the former Soviet Union presented a mix of the LAM and Beijing genotypes, while 1 isolate from Japan presented a mix of the EAI and Beijing genotypes.

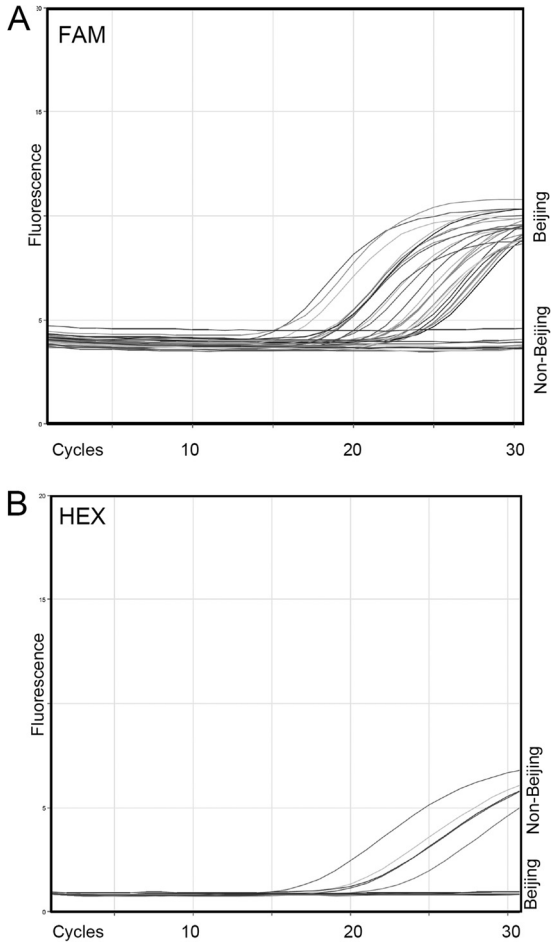

FIG 1 Fluorescence curves of a real-time PCR assay using DNA extracted from cultured bacteria. (A) Beijing genotype-specific signal (FAM channel, 510 nm); (B) other genotype-specific signal (HEX channel, 555 nm).

One hundred DNA samples extracted from clinical material (73 sputum samples, 8 bronchoalveolar lavage fluid [BALF] samples, and 19 other specimens, such as lung, tuberculoma, granulation, or chest cavity tissue) were randomly selected from the retrospective DNA collection. The concentration of DNA in the clinical specimens was estimated with a commercial kit to detect *M. tuberculosis* complex and was found to range from 4×10^2 to 1×10^6 copies per 10 μl. These specimens were subjected to the above-described real-time PCR under an increased number of cycles ($n = 50$). Clear-cut results were obtained for 86 of 100 samples, discriminating between Beijing ($n = 59$) and other genotypes ($n = 27$). PCR inhibition was checked using a control assay; as a result, no PCR inhibition was detected (data not shown). Thus, PCR failure (in 10 sputum and 4 BALF specimens) might have been due to the insufficient quantity/quality of the *M. tuberculosis* DNA.

A detailed discussion of different available methods for Beijing genotype detection (15–19) is beyond the scope of this short report. A larger prospective study should exploit the capacity of the developed method to identify the Beijing genotype in different kinds of clinical material. As a whole, a large-scale multicenter study is warranted to evaluate the different available PCR methods for detecting the Beijing genotype and for selecting those most suitable in terms of cost-effectiveness, performance, sensitivity, and specificity.

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